



UNIVERSITI PUTRA MALAYSIA

**EFFECTS OF PHYTIC ACID EXTRACTED FROM RICE BRAN ON
AZOXYMETHANE-INDUCED COLON CARCINOGENESIS IN RATS**

NORAZALINA SAAD

FPV 2008 13

**EFFECTS OF PHYTIC ACID EXTRACTED FROM RICE BRAN ON
AZOXYMETHANE-INDUCED COLON CARCINOGENESIS IN RATS**

By

NORAZALINA SAAD

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Master of
Sciences**

August 2008



Specially dedicated to

My mum and sister

For their invaluable love, understanding, encouragement and patience.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science

**EFFECTS OF PHYTIC ACID EXTRACTED FROM RICE BRAN ON
AZOXYMETHANE – INDUCED COLON CARCINOGENESIS IN RATS**

By

NORAZALINA SAAD

April 2008



Chairman : Norhaizan Mohd Esa, PhD

Faculty : Medicine and Health Sciences

This research is carried out to study the potential of phytic acid extracted from rice bran in the suppression of colon carcinogenesis in rats. In the optimization of phytic acid extraction, results showed 5% H₂SO₄ in pH 0.6 and 30 minutes of extraction time gave the highest amount of phytic acid. In animal study, 72 male *Sprague-Dawley* rats were divided into 6 groups with 12 rats in each group; Group 1: AOM alone, Group 2: AOM + 0.2% (w/v) Commercial Phytic Acid (CPA), Group 3: AOM + 0.5% (w/v) Commercial Phytic Acid (CPA), Group 4: AOM + 0.2% (w/v) Extract Phytic Acid (EPA), Group 5: AOM + 0.5% (w/v) Extract Phytic Acid (EPA). Rats received two subcutaneous injections of azoxymethane (AOM) in saline at (15mg/kg bodyweight) over a 2-weeks period to induce colon cancer. The treatments were given in two different concentrations of phytic acid; 0.2% (w/v) and 0.5% (w/v) during post initiation of carcinogenesis phase via drinking water.

The colons of the animals were analyzed for detection and quantification of aberrant crypt foci (ACF) after 8 weeks of treatment. The finding showed treatment with 0.2% (w/v) EPA gave the greatest reduction in the formation of ACF. In addition, phytic acid significantly suppressed the number of ACF in the distal, middle and proximal colon as compared to AOM alone ($p < 0.05$). For the histological classification of ACF, treatment with 0.5% (w/v) CPA had the highest percentage (71%) of non-dysplastic ACF followed by treatment with 0.2% (w/v) EPA (61%). After 20 weeks of treatment, colons of the rats were excised and analyzed for tumor incidence. Results showed that administration of phytic acid reduced the incidence and multiplicity of total tumors and adenocarcinomas even though there were no significant differences between groups.

For the immunohistochemical analyses, proliferating cell using Ki-67 and modulating of β -catenin and COX-2 expression were assessed as those have been shown to play a role in tumor progression. In Ki-67, there was a statistically significance difference in lowering the proliferating index between treatment groups as compared to AOM alone ($p < 0.05$). For β -catenin and COX-2 expression, there was a significant difference between groups as ($p = 0.000$) and ($p = 0.030$). In the correlation test, the results showed that there was a significant positive correlation ($p = 0.010$) between proliferation of Ki67 and COX-2 expression. A positive linear relationship was found between total Ki67 and β -catenin but these relationships were not statistically significant. Total β -catenin had a significant positive linear relationship with total COX-2 ($p = 0.044$).

As a conclusion, this study found the potential value of phytic acid extracted from rice bran in reducing colon cancer risk in rats. Besides identification of cancer reduction strategies based on dietary modification including looking at natural sources that may have anticancer properties, an alternative compound from local sources has been developed. Therefore, rice bran that is normally discarded as by-product of rice production will increase in value due to phytic acid potential as a nutraceutical compound in the prevention of colon cancer progression.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KESAN ASID FITIK YANG DIEKSTRAK DARIPADA DEDAK BERAS
TERHADAP TIKUS YANG TERARUH KANSER KOLON MENGGUNAKAN
AZOKSIMETANA**

Oleh

Norazalina Saad

April 2008

Pengerusi : Norhaizan Mohd Esa, PhD

Fakulti : Perubatan dan Sains Kesihatan

Kajian ini dijalankan dengan tujuan untuk mengkaji potensi asid fitik yang diekstrak daripada dedak beras dalam mengurangkan kejadian kanser kolon pada tikus. Dalam mendapatkan tahap pengekstrakan asid fitik, keputusan menunjukkan 5% H₂SO₄ dengan pH 0.6 dan masa pengekstrakan selama 30 minit telah memberikan jumlah amaun asid fitik yang tinggi. Dalam kajian haiwan eksperimen, 72 ekor tikus spesis *Sprague-Dawley* dibahagikan kepada 6 kumpulan dengan setiap kumpulan mempunyai 12 ekor; Kumpulan 1: Azoksimetana (AOM) sahaja, Kumpulan 2: AOM + 0.2% (w/v) Komersial Asid Fitik (CPA), Kumpulan 3: AOM + 0.5% (w/v) Komersial Asid Fitik (CPA), Kumpulan 4: AOM + 0.2% (w/v) Ekstrak Asid Fitik (EPA), Kumpulan 5: AOM + 0.5% (w/v) Ekstrak Asid Fitik (EPA), Kumpulan 6: Normal. Tikus telah menerima

dua suntikan AOM secara intraperitoneum dalam larutan garam yang steril pada kepekatan (15mg/kg berat badan) selama 2 minggu untuk mengaruh kolon kanser. Rawatan asid fitik diberi dalam dua kepekatan yang berbeza; 0.2% (w/v) dan 0.5% (w/v) selepas fasa permulaan karsinogenesis melalui air minuman.

Kolon daripada haiwan eksperimen dianalisa untuk mengesan dan mengira focus kript aberan (ACF) selepas rawatan selama 8 minggu. Keputusan menunjukkan bahawa rawatan dengan 0.2% (w/v) EPA memberikan kadar penurunan yang paling tinggi dalam pembentukan ACF. Selain itu, asid fitik secara signifikan merendahkan jumlah ACF pada kolon di bahagian distal, pertengahan dan proksimal dibandingkan dengan kumpulan AOM sahaja. Untuk pengelasan histologikal ACF, rawatan dengan 0.5% (w/v) CPA mempunyai peratus tertinggi (71%) ACF tisu yang tidak mengalami displasia diikuti oleh rawatan dengan menggunakan 0.2% (w/v) EPA (61%). Selepas rawatan selama 20 minggu, kolon tikus dikeluarkan dan dianalisa untuk melihat pembentukan tumor. Keputusan menunjukkan bahawa pemberian asid fitik boleh mengurangkan kejadian dan penggandaan jumlah tumor dan adenokarsinoma walaupun tidak terdapat perbezaan yang signifikan di antara kumpulan.

Untuk analisis menggunakan kaedah immunohistokimia, penentuan proliferasi sel Ki-67 dan modulasi ekspresi β -catenin dan COX-2 digunakan untuk menunjukkan peranan dalam perkembangan tumor. Dalam Ki-67, terdapat perbezaan yang signifikan dalam merendahkan indeks proliferasi antara

kumpulan rawatan apabila dibandingkan dengan AOM sahaja ($p < 0.05$). Dalam ekspresi β -catenin dan COX-2, terdapat perbezaan yang signifikan antara kumpulan ($p = 0.000$) dan ($p = 0.030$). Dalam ujian korelasi, keputusan menunjukkan terdapat korelasi positif yang signifikan ($p = 0.010$) antara perkembangan proliferasi Ki67 dan ekspresi COX-2. Didapati hubungan yang linear positif antara keseluruhan ekspresi Ki67 dan β -catenin, namun perkaitan adalah tidak signifikan. Keseluruhan β -catenin mempunyai hubungan linear positif yang signifikan dengan jumlah ekspresi COX-2 ($p = 0.044$).

Sebagai kesimpulan, kajian ini menunjukkan nilai potensi asid fitik yang diekstrak daripada dedak beras dalam mengurangkan risiko kanser kolon pada tikus. Selain penentuan strategi untuk mengurangkan kanser berdasarkan modifikasi diet dengan memberi perhatian kepada sumber semula jadi yang mempunyai sifat anti kanser, sebatian alternatif daripada sumber tempatan dapat dihasilkan. Dengan itu, dedak beras yang biasanya dibuang sebagai hasil sampingan dalam penghasilan beras akan dapat ditingkatkan nilainya dengan adanya asid fitik yang berpotensi sebagai agen nutraseutikal dalam pencegahan perkembangan kolon kanser.

ACKNOWLEDGEMENTS

In the name of Allah, the most benevolent and most merciful. I would like to take this opportunity to thank all those who gave great support to me while doing this thesis. First and foremost, I would like to extend my deepest gratitude to my supervisor, Dr. Norhaizan Mohd Esa, for her generous guidance, advice and endless support that contributed significantly towards the completion of this project. Her careful reviews and constructive criticism have been crucially important for this thesis.

My sincere gratitude is also accorded to my co-supervisors, Assoc. Prof. Dr. Hairuszah Ithnin and Prof. Dr. Maznah Ismail for their constructive advice, priceless comment and invaluable advice throughout the entire course of this research.

I gratefully acknowledge staff, laboratory assistants at Department Nutrition and Dietetics especially Mrs. Siti Muskinah, Mrs. Maznah and Mr. Simon for their help and cooperation. I would also like to acknowledge all the staff of Histopathological Laboratory, especially Mrs. Juita, Miss Zamzurina and Mrs. Normah for their assistance and kindly allowing me to access their laboratory facilities.

Special thanks are expressed to Dr. Ahmad Bustamam for allowing me to use Laboratory of Cancer Research MAKNA-UPM, Institute of Biosciences for immunohistochemical work.

I am indebted to my friends, especially Mr Muhammad Taib, Mrs. Shana, Ms. Azura, Ms. Sareena, Ms Nurzillah, Mrs. Nurhafzan Anis, Mrs. Kamilah, Mrs. Azizah, Ms. Tahirah, Mr. Nazmi, and Mr. Yip Wai Kien for their collaboration and continuous support and perseverance.

Finally, yet importantly I would like to express my heartiest appreciation to all my family members for their understanding and support throughout my studies.

Above all, I thank God for His mercy and blessing on me.



I certify that an Examination Committee has met on 27 August 2008 to conduct the final examination of Norazalina Saad on her Master of Science thesis entitled "Effects of Phytic Acid Extracted from Rice Bran on Azoxymethane-Induced Colon Carcinogenesis in Rats" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

Fauziah Othman, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Asmah Rahmat, PhD


Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sabariah Abdul Rahman, MBBS, M. Path

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Azian Abdul Latiff, PhD

Associate Professor
Faculty of Medicine
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)


HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 November 2008

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Norhaizan Mohd Esa, PhD

Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Hairuszah Ithnin, MD, M. Path

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Maznah Ismail, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

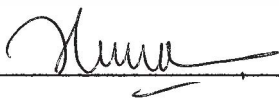


HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 December 2008

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institution.



NORAZALINA SAAD

Date: 28.10.2008

TABLE OF CONTENTS

	Page
DEDICATION	i
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	viii
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xx
 CHAPTER	
 1 INTRODUCTION	
1.1 Research Background	1
1.2 Importance of Study	4
1.3 Objectives	7
1.3.1 General Objective	7
1.3.2 Specific Objectives	7
1.4 Null Hypotheses	8
 2 LITERATURE REVIEW	
2.1 Introduction to phytic acid	9
2.1.1 Potential positive roles of phytic acid	12
2.2 Rice bran	16
2.2.1 Health benefits of rice bran	21
2.3 Carcinogenesis	22
2.3.1 Cancer	24
2.3.2 Chemical carcinogens	28
2.4 Colon Carcinogenesis	30
2.4.1 Anatomy and physiology of colon	30
2.4.2 Cell proliferation	31
2.4.3 Aberrant crypt	32
2.4.4 Adenomatous polyps	35
2.4.5 Colorectal carcinoma	37
2.4.6 Genetic alteration define in growth colorectal adenoma/carcinoma	39
2.5 Anti-cancer effects of phytic acid	46
2.5.1 Phytic acid as a therapeutic and preventive agent for cancer	51
2.5.2 Mechanism of cancer suppression	54
2.6 Analysis of phytic acid in foods	56



3	MATERIALS AND METHODS	
3.1	Materials	
3.1.1	Rice Bran	59
3.1.2	Chemicals and reagents	59
3.1.3	Instruments and Equipments	61
3.2	Methods	
3.2.1	Stabilization of Rice Bran	62
3.2.2	Lipid Extraction	62
3.2.3	Optimization of phytic acid extraction from rice bran	63
3.2.4	Spectrophotometrical determination of phytic acid	63
3.2.5	Neutralisation of phytic acid	64
3.2.6	Anion-Exchange purification	65
3.2.7	HPLC method for phytic acid analysis	66
3.3	<i>In vivo</i> study	
3.3.1	Animals	66
3.3.2	Ethical approval	67
3.3.3	Carcinogen treatment	67
3.3.4	Experimental design	67
3.3.5	Termination of experiment	68
3.3.6	Histological analysis	70
3.3.7	Immunohistochemistry	75
3.4	Statistical Evaluation	79
4	RESULTS	
4.1	Optimization of phytic acid extraction from rice bran	80
4.2	Spectrophotometrical determination of phytic acid	81
4.3	Separation of phytic acid using HPLC	
4.4	<i>In vivo</i> bioassay	83
4.4.1	Body Weights and Water Consumption	84
4.4.2	Effect of phytic acid on the incidence of Aberrant Crypt Foci (ACF) in rat colon induced by AOM	92
4.4.3	Histological Classification of ACF	100
4.4.4	Tumor Assessment	119
4.4.5	Immunohistochemical Staining of Ki-67, β -catenin and COX-2 in colonic tissue	109
5	DISCUSSION	
5.1	Optimization of phytic acid extraction from rice bran	118
5.2	Spectrophotometrical determination of phytic acid	122
5.3	Neutralization of phytic acid extract	123
5.4	Anion-exchange purification	124
5.5	Separation of phytic acid using HPLC	128
5.6	<i>In vivo</i> study	128
5.6.1	Effect of phytic acid on the incidence of Aberrant	128

	Crypt Foci (ACF) in rat colon induced by AOM	
5.6.2	Histological Classification of ACF	132
5.6.3	Tumor Assessment	132
5.6.4	Immunohistochemical Staining of Ki-67, β -catenin and COX-2 in colonic tissue	138
5	CONCLUSION AND RECOMMENDATION	148
6	REFERENCES	151
	APPENDICES	180
	BIODATA OF STUDENT	183

LIST OF TABLES

Table	Page
2.1 Phytic acid content (g/100g) of various cereals, root and fruit vegetables	9
2.2 Selected Agricultural Production ('100 tones)	15
2.3 Nutrient Composition of Stabilised Full Fat Rice Bran	18
2.4 Comparison of rice bran to other cereal brans	19
2.5 Characteristic of aberrant crypt	33
2.6 Variables affecting the utility of the ACF system	33
2.7 Antitumor effect of inositol hexaphosphate (IP6) <i>in vitro</i>	47
2.8 Antitumor effect of IP6 and inositol <i>in vivo</i>	51
3.1 Tissue dehydration in a tissue processor machine	72
3.2 Staining with Haematoxylin and Eosin (H&E)	73
3.3 Histological criteria of ACF classification	74
3.4 Semi-quantitative scoring system for evaluation of immunohistochemical staining	80
4.1 Comparison of phytic acid amount in three different extracting solvents by optimization of different condition of extraction	81
4.2 The changes of water consumption throughout the experiment	100
4.3 The effect of phytic acid on the incidence of ACF in rat colon induced by azoxymethane (AOM)	103
4.4 Regional distribution of colonic ACF in rats treated with AOM and fed with phytic acid	107
4.5 Histological classification of ACF	114
4.6 Tumor Assessment	120



4.7	Mean score for the expression of Ki-67, β -catenin and COX-2 in colonic tissue	127
4.8	Comparison between groups of treatment in Ki-67 scores	129
4.9	Comparison between groups of treatment in β -catenin scores	131
4.10	Comparison between groups of treatment in COX-2 scores	133
4.11	Correlation among the total scores of Ki-67, β -catenin and COX-2	134

LIST OF FIGURES

Figure	Page
2.1 Structure of phytic acid (myo-inositol hexaphosphate or IP6)	8
2.2 Rice kernel structure	16
2.3 Multistage process of cancer	23
2.4 The division of cancer cell and two healthy white blood cells	24
2.5 Series of mutations whereby each mutation alters the behavior of the cell	26
2.6 Possible pathway of activation and inactivation of DMH	28
2.7 Anatomy and view of layers of the colon	29
2.8 Detail histology of colon	30
2.9 Histological features of two ACF	34
2.10 Microscopic appearance of a pedunculated tubulovillous adenoma, polystratification of cells and moderate dysplasia are evident and villous adenoma	36
2.11 Correlation between histological progression and molecular changes in colorectal cancer	39
2.12 Overview of the Wnt pathway	40
2.13 Summary of eicosanoids	43
2.14 Summary of the prostanoid synthesis	44
3.1 Experimental design of <i>in vivo</i> study	70
3.2 Photomicrography of normal crypt and ACF	72
4.1 Calibration curve of phytic acid	86
4.2 Separation of phytic acid using HPLC	92
4.3 Changes in body weight of experimental rats	95



4.4	Final liver, kidney and heart weight after 8 weeks treatment with phytic acid	97
4.5	Final liver, kidney and heart weight after 20 weeks treatment with phytic acid	98
4.6	Effect of phytic acid on azoxymethane-induced colonic aberrant crypt foci (ACF) in Sprague-Dawley rats	104
4.7	Histological examination using H&E staining of aberrant crypt foci of different sizes with surrounding normal tissue	105
4.8	Regional distribution of colonic ACF in rats treated with AOM and fed with phytic acid	108
4.9	Morphological evaluations of ACF in different groups of rats were assessed for the presence and degree of dysplasia using H&E staining	116
4.10	Histopathology of colonic lesions developed in rat group treated with AOM	122
4.11	Immunohistochemical staining of Ki-67, β -catenin and COX-2 in a few groups of rats	128

LIST OF ABBREVIATIONS

AEC	Anion-exchange chromatography
ACF	Aberrant crypt foci
AM	Azomethane
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemist
AOM	Azoxymethane
APC	Adenomatous polyposis coli
APES	3-aminopropyltrimethoxysilane
BERNAS	Padi Beras Nasional Berhad
BSA	Bovine Serum Albumin
°C	Degree celsius
Ca ²⁺	Calcium
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CPA	Commercial phytic acid
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DMH	Dimethylhydrazine
DNA	Deoxyribonucleic acid
DPX	di-n-butylphthalate-polystyrene-xylene
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EPA	Extract phytic acid
FAP	Familial adenomatous polyposis coli
FFA	Free fatty acids
Fe ²⁺	Ferrous ion
Fe ³⁺	Ferric ion
g	Gram
g/ml	Gram/mililiter
GSK3 β	Glycogen synthase kinase
H ⁺	Hydrogen ion
H & E	Hematoxylin and eosin
H ₂ O	Water
H ₂ SO ₄	Sulphuric acid
HCl	Hydrochloric acid
HNPCC	Hereditary non-polyposis colorectal cancer
HPLC	High Performance Liquid Chromatography
hrs	Hours
IP3	Inositol triphosphate
IP4	Inositol tetraphosphate
IP5	Inositol pentaphosphate
IP6	Inositol hexakisphosphate
InSP ₃	Inositol 1,4,5-trisphosphate
IL1	Interleukin-1

IL2	Interleukin-2
K ⁺	Kalium
Kcal	Kilocalorie
M	Molar
MAM	Methylazoxymethanol
MD	Methyldiazonium
Mg ²⁺	Magnesium ion
MHz	Megahertz
µg	Microgram
mg	Miligram
ml	Milliliter
mM	Milimolar
mm ²	Millimeter square
NaCl	Natrium chloride
NaOH	Natrium hydroxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OH ⁻	Hydroxide ion
O ₂	Oxygen
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PCNA	Proliferating Cell Nuclear Antigen
PGI ₂	Prostacyclin
PGE ₂	Prostaglandin E ₂

PGD2	prostaglandin D2
PGH2	Prostaglandin H2
PGs	Prostaglandins
pH	Per hydrogen
RD	Rhabdomyosarcoma
SD	Standard deviation
SPSS	Statistical Package for the Social Science
TBS	Tris Base Saline
TBST	Tris-buffered saline with Tween-20
TCA	Trichloroacetic acid
Tcf	T cell factor
TNF	Tumor necrosis factor
TxA2	Thromboxane
UV	Ultraviolet
UVB	Ultraviolet light B
v/v	Volume/volume
WHO	World Health Organization
w/v	Weight/volume

